APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2008, p. 3839–3848 Vol. 74, No. 12
0099-2240/08/$08.00+0 doi:10.1128/AEM.02343-07
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Temporal Variations in the Dynamics of Potentially Microcystin-Producing Strains in a Bloom-Forming *Planktothrix agardhii* (Cyanobacterium) Population▽†

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Received 17 October 2007/Accepted 18 April 2008

The concentration of microcystins (MCs) produced during blooms depends on variations in both the proportion of strains containing the genes involved in MC production and the MC cell quota (the ratio between the MC concentration and the density of cells with the *mcyA* genotype) for toxic strains. In order to assess the dynamics of MC-producing and non-MC-producing strains and to identify the impact of environmental factors on the relative proportions of these two subpopulations, we performed a 2-year survey of a perennial bloom of *Planktothrix agardhii* (cyanobacteria). Applying quantitative real-time PCR to the *mcyA* and phycocyanin genes, we found that the proportion of cells with the *mcyA* genotype varied considerably over time (ranging from 30 to 80% of the population). The changes in the proportion of cells with the *mcyA* genotype appeared to be inversely correlated to changes in the density of *P. agardhii* cells and also, to a lesser extent, to the availability of certain nutrients and the abundance of cladocerans. Among toxic cells, the MC cell quota varied throughout the survey. However, a negative correlation between the MC cell quota and the *mcyA* cell number during two short periods characterized by marked changes in the cyanobacterial biomass was found. Finally, only 54% of the variation in the MC concentrations measured in the lake can be explained by the dynamics of the density of cells with the MC producer genotype, suggesting that this measurement is not a satisfactory method for use in monitoring programs intended to predict the toxic risk associated with cyanobacterial proliferation.

Cyanobacteria in freshwater ecosystems are a source of growing concern, because their proliferation leads to ecological disturbances and the toxins they produce constitute health risks for animals as well as human beings (4, 24). These microorganisms are known to produce numerous bioactive secondary metabolites (reviewed in reference 62), which can be toxic (64). These metabolites include microcystins (MCs), which are the most commonly occurring cyanotoxins and have been involved in several poisonings of animals and human beings (7). MCs are hepatotoxic cyclic heptapeptides (for their general structure and nomenclature, see reference 6) synthesized nonribosomally by a multifunctional enzyme complex (13). The gene cluster coding for this enzyme complex consists of 9 or 10 genes, depending on the genus. The corresponding genes of *Microcystis aeruginosa* K-139 and PCC 7806 (34, 53), *Planktothrix agardhii* CYA 126 (9), and *Anabaena* sp. strain 90 (44) have been completely sequenced.

The concentration of MCs in cells or in the water during a cyanobacterial bloom cannot be predicted. This is partly because of differences in the proportions of MC-producing and non-MC-producing subpopulations within cyanobacterial populations (27, 58, 59) and partly because of toxin heterogeneity within an MC-producing subpopulation (65) and/or differences at the level of the expression of the genes involved in the biosynthesis of these molecules (16, 20). One of the most interesting questions regarding MCs concerns the influence of environmental factors and processes on the selection of the MC-producing subpopulation and on the expression of genes involved in MC biosynthesis.

Several previous studies have investigated the influence of environmental factors, such as light and nutrients, on MC production by toxic cyanobacterial strains (e.g., references 5, 14, 36, 40, 41, 49, 55, 57, 60, and 63). Despite rather inconsistent findings, in general MC production seems to be linked to the growth rate of the cells, which in turn depends on environmental conditions (5, 15, 29, 30, 37, 39, 50).

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 25 April 2005.

With regard to the selection of MC-producing and non-MC-producing subpopulations, a few recent studies using real-time PCR or competitive PCR to amplify some of the genes involved in MC production (*mcy* genes) have studied the dynamics of these two subpopulations during cyanobacterial blooms. For the genus *Microcystis*, the proportion of putative MC-producing strains appears to be low (0 to 30%) (25, 66) and to depend on nitrate concentrations (67) or, more generally, on the trophic status of the habitat (38). Despite the widespread occurrence of toxic *Planktothrix* blooms in freshwater ecosystems, less information for this genus is available. However, characterizations of isolated strains have suggested that the proportion of MC-producing strains in *Planktothrix* populations is higher than that in *Microcystis* populations (27, 65).

Against this general background, this study was intended to determine (i) the subpopulation dynamics of MC-producing strains (the subpopulation containing *mcy* genes) and non-MC-producing strains (those with genotypes lacking the *mcy* genes) during a *P. agardhii* bloom, (ii) the impact of environmental factors and processes on the proportion of strains with the *mcyA* genotype, and finally, (iii) the relationship between differences in densities of cells with the *mcyA* genotype and differences in MC concentrations. We focused our studies on a perennial bloom of *P. agardhii* that has been occurring since 1999 in an artificial, shallow, eutrophic French lake, the Base Nautique de Viry (BNV), which is typical of several lakes in Paris suburban areas (3, 65).

MATERIALS AND METHODS

Study site. Sampling was performed at the BNV in a suburban area of Paris, France (2°23′04.2″E, 48°40′03.3″N). The BNV is a shallow artificial lake (mean depth, 2.8 m; area, 98 ha) that receives both diffuse and point source inputs from an 810-ha watershed. The main point source of input is a rainfall collector, located on the south shore of the lake, which collects untreated surface water from a 430-ha subbasin. In December 2003, a treatment plant was added to the rainfall collector to improve the quality of the outflow water released into the BNV. This treatment comprises filtration (through 3-mm pores), flotation, and decantation to limit the discharge of suspended solids. The lake is a recreational area (used for fishing, sailing, diving, and water skiing). The water body is the site of a perennial MC-producing development of *P. agardhii* (3, 65). *Limnothrix redekei* has also been observed in this lake.

Sampling. In view of the size of the lake and the absence of thermic stratification, sampling was conducted at one sampling station twice monthly from 23 March 2004 to 21 March 2006. On each sampling occasion, three replicate samples were taken from 0.5 m below the water surface.

The water temperature was measured every 10 min during the entire study period by using an in situ thermistor chain (four Seamon Mini electronic temperature recorders with a reported accuracy of within 0.05°C; Hugrun Inc., Reykjavik, Iceland). The average surface temperature of the lake over the period between two sampling dates was then calculated. The dissolved-oxygen concentration and the conductivity on each sampling date were measured by using a multiparameter SBE 19 Seacat profiler (Sea-Bird Electronics, Inc., Bellevue, WA). Transparency was measured with a Secchi disk.

To evaluate the dissolved nutrients, the water was filtered on site by using a cellulose acetate 0.22-μm-pore-size syringe filter (Nalgene, Rochester, NY), kept refrigerated in acid-washed polyethylene containers, and analyzed the same day. Soluble reactive phosphorus (SRP) and ammonia (NH_4^+) concentrations were determined on the day of sampling by a spectrophotometric method as described previously (11). Detection limits were 1 μM for NH_4^+ and 0.1 μM for SRP. The nitrate (NO_3^-) concentration was measured using a DX600 ion chromatograph (Dionex Corp., Westmont, IL) equipped with an AS14 IonPack analytical column (Dionex). The threshold of detection was 0.85 μM. Total phosphorus and total nitrogen levels were measured according to standard NF EN ISO 6878 (2) by using unfiltered water which was poured into acid-washed polyethylene containers.

For chlorophyll *a* assays, 300 ml of water was collected on Whatman GF/C filters. Concentrations were determined using a Cary 50 scan spectrophotometer (Varian Inc., Palo Alto, CA) after methanol extraction (51) and expressed as micrograms per liter.

Aliquots (20 ml) of the water samples were fixed immediately with formaldehyde (5% final concentration [vol/vol]) for phytoplankton species determination. *P. agardhii* cell densities were determined using a Malassez counting chamber with an Optiphot 2 microscope (magnification, ×400; Nikon, Melville, NY) or Utermöhl chambers (31) with an inverted CK2 microscope (magnification, ×400; Olympus Optical Co., Tokyo, Japan) when the phytoplanktonic cell density was low. At least 400 trichomes were counted to reduce the error to less than 10% ($P = 0.95$) (19). Trichome counts were converted into cell counts, assuming that a 100-μm trichome unit contained 25 cells (standard deviation, 5 cells).

Zooplankton was collected by filtering 1.5 liters of water across a 60-μm-pore-size mesh and preserved in ethanol (70%) (12). Abundances (individuals [ind] per liter) were estimated using an inverted microscope (CK2; Olympus, Tokyo, Japan) after sample sedimentation. Three major taxonomic groups were distinguished: rotifers, cladocerans, and cyclopoid copepods. The last group was differentiated according to the different stages of development: nauplii and copepodites plus adults. Calanoid copepods were not taken into consideration because of their low abundance (<10 ind liter^{-1}).

Strains for primers, probes, and PCR testing. Strains used for primers, probes, and PCR testing were maintained in culture in the Paris Museum Collection (65). Several strains isolated previously from the BNV were either MC-producing strains, such as *P. agardhii* PMC 51.02, PMC 74.02, PMC 75.02, PMC 86.02, PMC 109.02, and PMC 212.02, or non-MC-producing strains, such as *P. agardhii* PMC 87.02 and PMC 101.02 and *L. redekei* PMC 85.02 (65). The MC-producing *Microcystis* strain PCC 7820 was also used for *mcyA* gene testing.

DNA extraction. For each sampling date, a 400- or 600-ml sample of water was centrifuged (for 10 min at 3,220 × *g*) to obtain 20 ml of a concentrated solution. DNA was extracted from 2.5-ml aliquots of these solutions by using the DNeasy tissue kit (Qiagen) according to the manufacturer's protocol for DNA extraction from gram-negative bacteria. Briefly, cells were lysed for 3 h with proteinase K, and DNA was eluted off the column in two steps by using 100 μl and then 50 μl of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0; Qiagen). The concentration and the purity of the DNA extracted were determined using a spectrophotometer (Uvikon 922; Kontron Instruments) at 260 and 280 nm. The DNA was stored at −20°C until being subjected to real-time PCR analysis.

The influence of the DNA extraction methods on the sensitivity of the real-time PCR was tested. For both genes studied (the phycocyanin [PC] operon and *mcyA*), the same regression equations for two MC-producing *P. agardhii* strains, PMC 75.02 and PMC 86.02, were obtained after phenol-chloroform extraction (26) and after extraction by the DNeasy tissue kit (Qiagen) method (data not shown), showing that our real-time PCR results, unlike those of Schober and Kurmayer (48), did not depend on the DNA extraction method used.

Primer design and multiplex real-time PCR. The proportion of *mcy* genotypes in the *Planktothrix* population was determined by real-time PCR analysis. Two target gene regions were used: the intergenic spacer region within the PC operon and the *mcyA* region. Our nucleotide amplicon corresponded to a peptide sequence located at the end of the condensation domain and the beginning of the adenylation domain A2 (9), which carries out one step in MC biosynthesis (9). PC gene amplification was used to check the validity of each multiplex PCR and to target both *mcyA*- and non-*mcyA*-containing *Planktothrix* subpopulations. Several sequences of PC and *mcyA* genes publicly available in databases were aligned comparatively with those of the MC-producing *Planktothrix* strain NIVA-CYA 126/8 (9) and the MC-producing *Microcystis* strain PCC 7806 (53) by using GeneDoc software (33). Primers and TaqMan probes used in this study (listed in Table 1) were newly designed using Primer3 Input software (45). The sequence specificities of the primers and probes were checked by a BLAST search of the NCBI databases. The specificities were confirmed by amplification tests with MC-producing strains *P. agardhii* PMC 51.02, PMC 74.02, PMC 75.02, PMC 86.02, PMC 109.02, and PMC 212.02 and *Microcystis* strain PCC 7820 and with non-MC-producing strains *P. agardhii* PMC 87.02 and PMC 101.02 and *L. redekei* PMC 85.02. The PC gene fragment was amplified in all *P. agardhii* strains, whereas the *mcyA* gene fragment was amplified only in the six MC-producing *P. agardhii* strains (data not shown).

Amplification by real-time PCR was carried out using an Mx3000P thermal cycler (Stratagene, Amsterdam, Netherlands). All the reactions were performed with 20-μl volumes in 96-well plates (Stratagene). The multiplex reaction mix contained 10 μl of 2× QuantiTec Probe PCR Master Mix (Qiagen), 0.6 μl of each primer (10 μM; Eurogentec), 0.4 μl of each TaqMan probe (10 μM; Eurogentec), 4.8 μl of sterile Millipore water, and 2 μl of the diluted DNA template. Each sample was prepared in duplicate. Negative controls without DNA and positive controls comprising the genomic DNA of the strain *P. agardhii*

TABLE 1. Real-time PCR primers and probes used in this study

Gene region and primer or probe	Sequence[a] (5′–3′)
mcyA region	
MAPF	CTAATGGCCGATTGGAAGAA
MAPR	CAGACTATCCCGTTCCGTTG
MAP Taq	FAM-CTCTGCGGTTACAGCTAACGGGTGG-BHQ-1
PC gene region	
FPC1	AACCCATAGGGAGATAACTC
RPC1	GCTTTGGCTTGACGGAAACG
PPC Taq	CYA-ATTCTCAAGGCCGTTTCCTGAGCAG-BHQ-1

[a] FAM, 6-carboxyfluorescein; BHQ-1, black hole quencher-1; CYA, 5-indocarboxycyanine.

PMC 75.02 were included for each PCR run. The temperature cycle consisted of an initial preheating step of 15 min at 95°C, followed by 38 cycles of 15 s at 95°C (denaturing), 30 s at 60°C (annealing), and 30 s at 72°C (extension). For data analysis, the threshold for the fluorescence of all the samples was set manually to 317 (relative fluorescence) for PC gene amplification and to 480 for *mcyA* amplification in order to obtain the best PCR efficiency in linear-log calibration curves. The sizes of amplicons were 131 and 140 bp for the PC and *mcyA* genes, respectively.

MC analysis. Samples from the lake were filtered through Whatman GF/C filters. The cell-bound MC contents of the field samples were screened after methanol extraction by a protein phosphatase 2A (PP2A) inhibition bioassay as described previously (3). The toxin concentrations were calculated from a standard MC-LR calibration curve (Sigma Chemicals, Saint Quentin Fallavier, France). The results were expressed as microgram equivalents of MC-LR per liter. The detection limit was 0.1 μg equivalent of MC-LR liter^{-1} of raw water.

Data analysis. The principal component analysis (PCA) was performed using the ADE-4 software package (52), and the stepwise multiple regression (SMR) analysis was performed using the Statgraphics Centurion XV software (StatPoint Inc.).

RESULTS

Validation of the ΔC_T method applied to multiplex real-time PCR. We used a novel approach, the change in threshold cycle (ΔC_T) method, to estimate the relative proportion of the *mcyA* subpopulation in the *Planktothrix* population by a single multiplex real-time PCR.

Standard curves for the PC and *mcyA* genes were set up using the genomic DNA of six MC-producing *Planktothrix* strains, PMC 51.02, PMC 74.02, PMC 75.02, PMC 86.02, PMC 109.02, and PMC 212.02. For each strain, serial dilutions containing 40, 20, 4, 0.4, and 0.04 ng of the genomic DNA μl^{-1} were prepared. The standard curves were established by relating the known quantity of DNA (in nanograms) to the threshold cycle (C_T) number (the cycle number at which the fluorescence exceeds the threshold) for each diluted sample (see Fig. S1 in the supplemental material; Fig. 1A). For both genes, highly significant linear plots of the amounts of genomic DNA of the *P. agardhii* strains versus the C_T numbers were obtained. The regression equations, as well as the resulting ΔC_T between

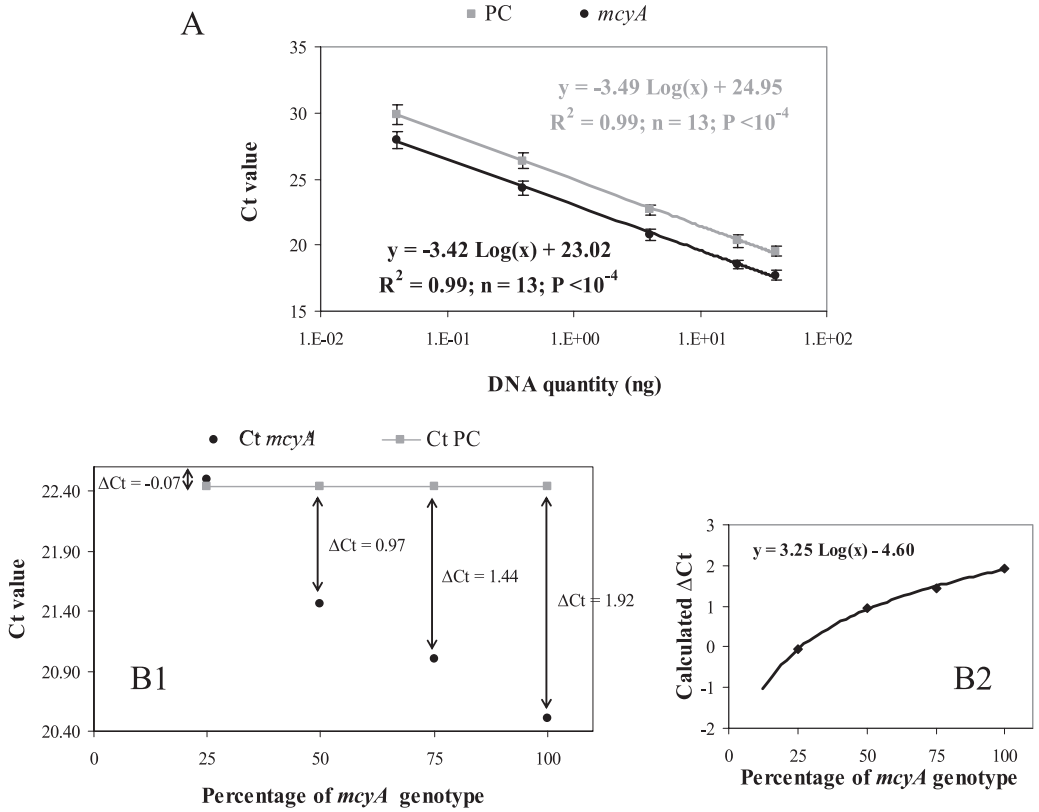

FIG. 1. (A) Example of the standard curves for the *mcyA* (black circles) and PC (gray squares) genes based on predetermined concentrations of DNA of the MC-producing *Planktothrix* strain PMC 75.02 amplified by multiplex real-time PCR. The error bars indicate standard deviations ($n = 13$). (B1) Example of C_T numbers obtained for the *mcyA* (black circles) and PC (gray squares) genes amplified by multiplex real-time PCR from a mix of MC-producing (PMC 75.02) and non-MC-producing (PMC 87.02) *Planktothrix* strains. (B2) The ΔC_T was calculated for each mix, and the ΔC_T equation was obtained by relating the calculated ΔC_T to the percentage of cells with the *mcyA* genotype.

the two genes ($\Delta C_T = C_T$ for the PC gene $- C_T$ for *mcyA*), are shown in Fig. S1 in the supplemental material. We found a narrow range of ΔC_T values (with a mean ΔC_T value of 1.94 ± 0.04) for the six MC-producing *Planktothrix* strains. This non-null value may be explained by differences between the two genes (the PC gene and *mcyA*) either in copy number or in amplification efficiency. However, knowing that the ΔC_T was the same for all the MC-producing strains tested and that the PC gene is present in each *P. agardhii* cell, we considered that the variations in the ΔC_T values for natural samples depend only on the proportion of the *mcyA* genotype. Therefore, a ΔC_T value of 1.94 obtained for a natural sample would indicate that the *Planktothrix* population consisted entirely of strains with *mcyA* genotypes. As the C_T decreases by 1 when the quantity of DNA decreases by 50%, the following equation was deduced from the ΔC_T values theoretically calculated for proportions of *mcyA* genotype strains of 100, 50, 25, and 12.5% of the population: $y = 3.32 \times \log(x) - 4.70$, where y is the ΔC_T and x is the initial percentage of *mcyA* genotype strains.

This theoretical ΔC_T equation was experimentally validated with samples containing a mix of the MC-producing strain PMC 75.02 and the non-MC-producing strain PMC 87.02. Four samples containing the MC-producing strain at 25, 50, 75, and 100% were tested, and the ΔC_T for each percentage was calculated (Fig. 1B1). The resulting ΔC_T equation [$y = 3.25 \times \log(x) - 4.60$] was similar to the theoretical ΔC_T equation [$y = 3.32 \times \log(x) - 4.70$] (Fig. 1B2), which validates our approach.

Furthermore, the ΔC_T results were compared to those of a more conventional method relying on the copy number of the gene or the number of cells (26). The standard curve for the gene copy number (GCN) method was established using 1 ml of a suspension containing 10^8 cells of the MC-producing strain PMC 75.02 ml^{-1} (as determined by a direct microscopic count). After DNA extraction, five dilutions of template DNA ranging from 1:10^1 to 1:10^5 (equivalent to 1.33×10^5 cells to 13.3 cells, respectively) were prepared. Equations relating the numbers of genes (PC and *mcyA* genes) to the C_T numbers for the diluted samples were obtained (see Table S1 in the supplemental material). Over the entire study period, a significant relationship between the *Planktothrix* cell numbers estimated by microscopic counting and those estimated by applying the GCN method to the PC gene was found (Fig. 2A). The regression equation was as follows: $y = 0.99x + 0.40$ (Pearson's $R^2 = 0.93$; $n = 45$; $P < 10^{-4}$), where y is the $\log_{10}$ of the cell number determined by the GCN method and x is the $\log_{10}$ of the microscopic cell count. The ΔC_T method and the GCN method were then applied to the same data set of multiplex real-time PCR results obtained from the 2-year BNV sampling. A comparison of the methods for estimating the proportion of cells with the *mcyA* genotype in the *P. agardhii* population showed the results of the two methods to be very significantly correlated (Pearson's $R^2 = 0.92$; $n = 45$; $P < 10^{-4}$) (Fig. 2B).

Temporal patterns of the dynamics of the *P. agardhii* population and of the proportion of the *mcyA* genotype strains. During this 2-year survey, the *Planktothrix* population dominated the phytoplankton community in the BNV during two periods (Fig. 3A). Moreover, a significant negative linear correlation (Pearson's $R^2 = 0.42$; $n = 45$; $P < 10^{-4}$) between the *P. agardhii* cell densities and the proportions of the *mcyA* genotype strains was observed.

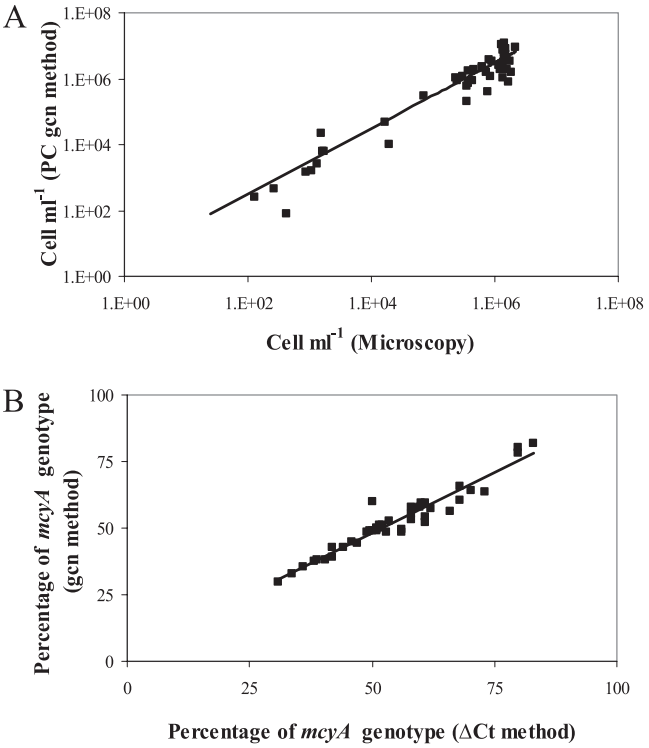

FIG. 2. Correlations between the *Planktothrix* cell densities in the BNV from March 2004 to March 2006 as determined by counting under the microscope and as determined by the GCN method for the PC gene (PC gcn) (A) and between the results of the GCN and ΔC_T methods used to evaluate the percentage of cells with the *mcyA* genotype (B).

A more detailed analysis revealed four distinct periods (Fig. 3A). In the first period, extending from March to December 2004, the *P. agardhii* cell density ranged from 1.14×10^6 to 2.15×10^6 cells ml^{-1}, and *P. agardhii* dominated the phytoplankton community (accounting for 93% of the population over this period). Meanwhile, the proportion of the *mcyA* genotype strains ranged from 31 and 61%, with a mean value of 46%. The second period, lasting from December 2004 to May 2005, appeared to be a transition period during which the *P. agardhii* cell density fell steadily from 1.4×10^6 to 25 cells ml^{-1}. Toward the end of this period (from 11 March to 1 May 2005), *L. redekei* dominated the phytoplankton community, accounting for up to 76% of the population. The density of *L. redekei* cells never exceeded 4.3×10^5 cells ml^{-1}. During the second period, the proportion of the *mcyA* genotype strains in the *Planktothrix* population increased from about 36 to 60%. The third period, extending from May to October 2005, was characterized by low densities of *P. agardhii* cells of between 25 and 2×10^4 cells ml^{-1} and high proportions of *mcyA* genotype strains, ranging from 56 to 73%. During the fourth period, extending from November 2005 to March 2006, the *P. agardhii* cell density rose from 7.3×10^4 to 7.8×10^5 cells ml^{-1} and *P. agardhii* once more dominated the phytoplankton community (accounting for 88% of the population over this period). Moreover, the proportion of *mcyA* genotype strains ranged from 52 to 83%, with a mean value of 65%. During this fourth period, the highest proportions of *mcyA* genotype strains (≥80%) were

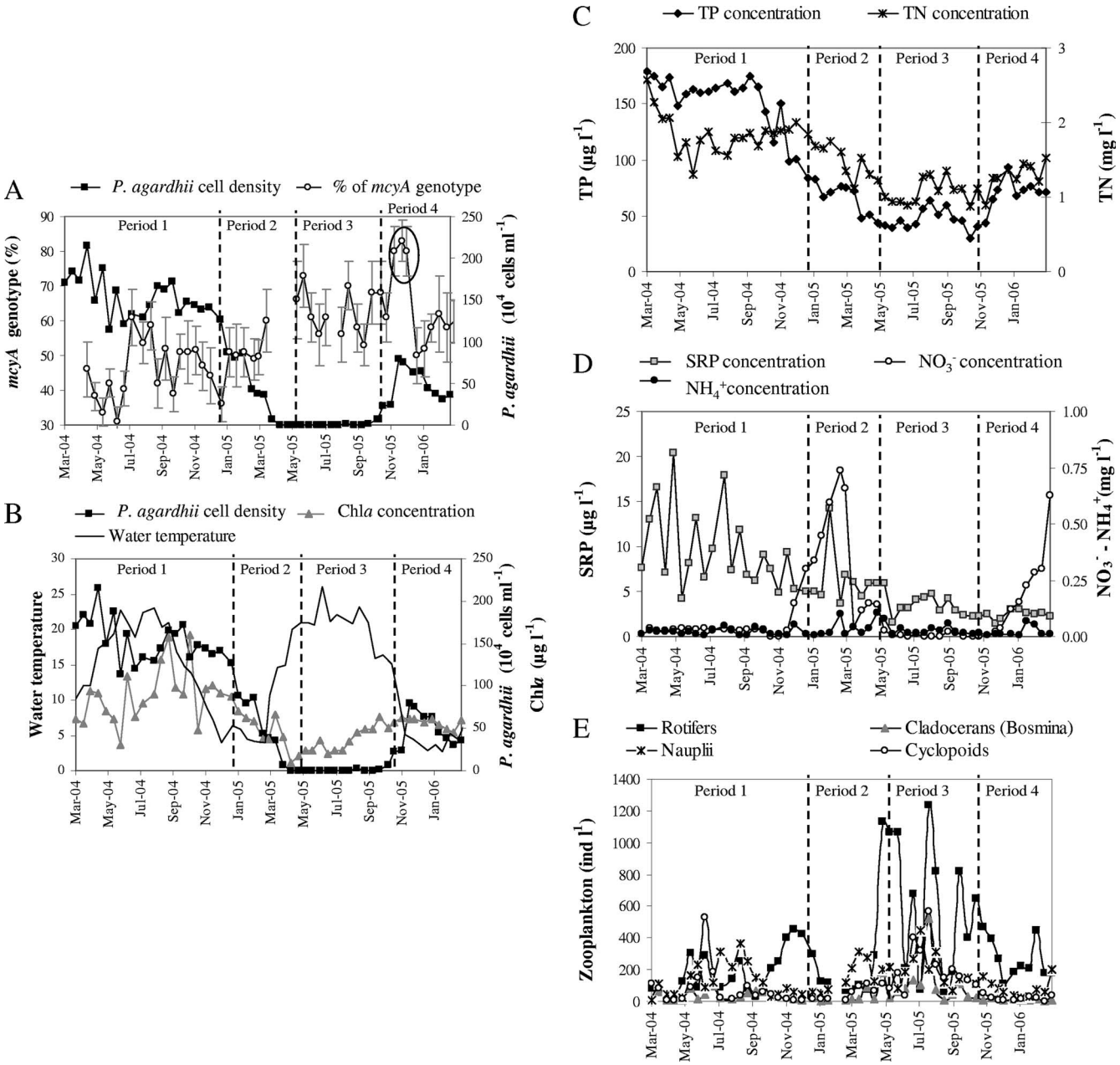

FIG. 3. Phytoplankton, zooplankton, and environmental parameters for the lake from March 2004 to March 2006. (A) Changes in the density of *P. agardhii* cells and in the proportion of *P. agardhii* cells with the *mcyA* genotype. The highest proportions of cells with the *mcyA* genotype are circled. The error bars indicate standard deviations ($n = 4$). (B) Densities of *P. agardhii* cells, chlorophyll *a* (chl*a*) concentrations, and water temperatures. (C) Total phosphorus (TP) and total nitrogen (TN) concentrations. (D) SRP, ammonium (NH_4^+), and oxidized nitrogen (NO_3^-) concentrations. (E) Abundance of the main groups of zooplankton. The four periods are separated by dashed lines.

obtained on three sampling dates, 29 November and 13 and 23 December 2005 (Fig. 3A), and were concomitant with the increased abundance of *P. agardhii* cells.

The dynamics of the *mcyA* genotype strains are related to environmental factors. Changes in several environmental factors during this study were recorded (Fig. 3). Water temperatures ranged between 2.7 and 26°C (Fig. 3B), with temperatures of over 19°C for 4 months (from June to September) in each year studied. Chlorophyll *a* concentrations ranged from a minimum value of 9.9 μg liter^{-1} in spring 2005 to a maximum value of 159.6 μg liter^{-1} in fall 2004 (Fig. 3B). There was a significant correlation (Pearson's $R^2 = 0.49$; $n = 52$; $P < 10^{-4}$) between the concentration of chlorophyll *a* and the *P. agardhii* cell density.

Changes in the total phosphorus and total nitrogen concentrations (Fig. 3C) were closely correlated to the changes in *P. agardhii* cell density (Pearson's $R^2 = 0.85$; $n = 52$; $P < 10^{-4}$; and Pearson's $R^2 = 0.72$; $n = 52$; $P < 10^{-4}$, respectively). The concentrations of SRP ranged from 1 to 20 μg liter^{-1} (Fig. 3D), the highest values being observed during the first period

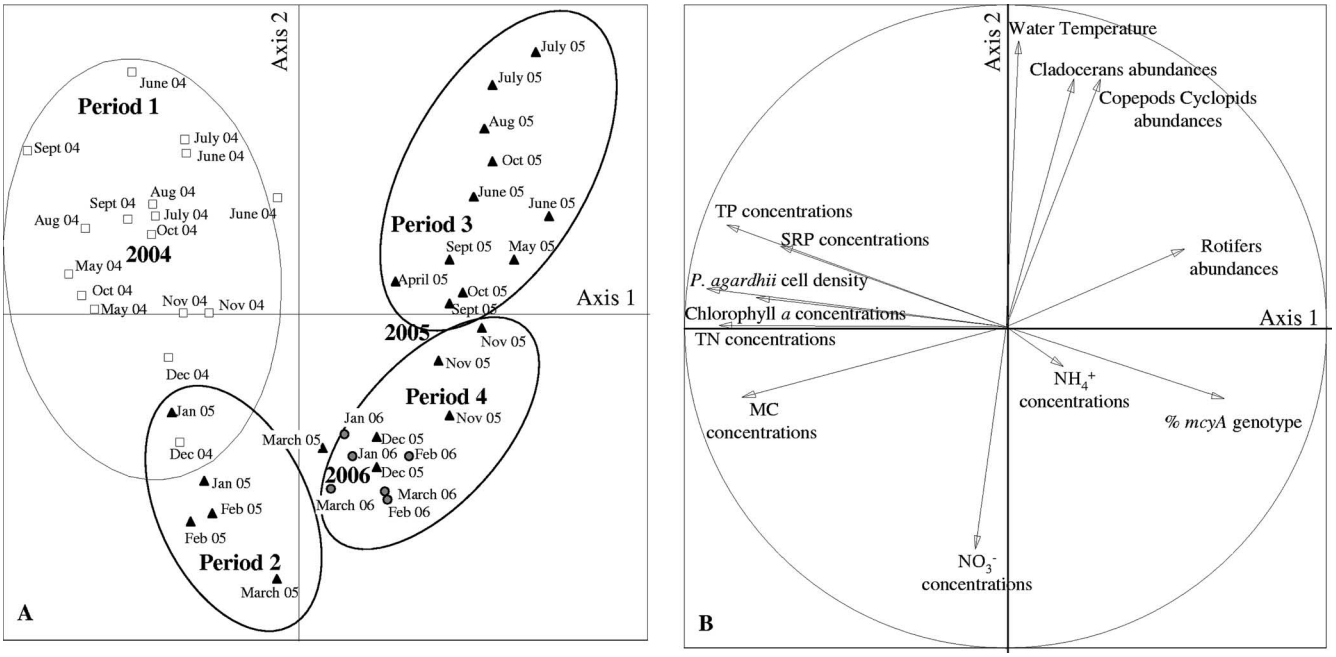

FIG. 4. Projection onto the plane defined by the two first axes of the PCA of the data from the 52 samples obtained between March 2004 and March 2006, which are divided into four groups (circled) (A), and the environmental and biological variables (B). The first two axes accounted for 60% of the total variability. TP, total phosphorus.

of *P. agardhii* development. Finally, the dissolved-nitrogen concentration was always low, except during brief increases in the nitrate concentration during the winters of 2005 and 2006, when the dissolved-nitrogen concentration reached 0.74 and 0.62 mg liter^{-1}, respectively (Fig. 3D).

The main zooplanktonic taxa collected from the BNV during the study were cyclopoid copepods, differentiated on the basis of the different stages of development (nauplii and copepodites plus adults), cladocerans (mainly from the genus *Bosmina*), and rotifers (Fig. 3E). Some changes were observed during the survey, but only the rotifers increased in abundance from April to November 2005 (the abundance peaked at 1,234 ind liter^{-1} in August), when the density of *P. agardhii* cells decreased.

A PCA of the whole data set (including data for 52 samplings, 10 environmental variables, the proportion of the *mcyA* genotype strains, the *P. agardhii* cell density, and the MC concentration) was performed (Fig. 4). The first two axes of this PCA accounted for more than 60% (39.4% plus 21.5%) of the total variation. When the sampling dates (Fig. 4A) were projected onto the plane defined by the first two axes, these dates could be structured into four groups corresponding to the four periods defined above: period 1 from May to December 2004, period 2 from December 2004 to May 2005, period 3 from May to October 2005, and period 4 from November 2005 to March 2006. Projecting the variables onto the same plane (Fig. 4B) showed that total phosphorus, total nitrogen, and SRP concentrations, *P. agardhii* cell densities, and chlorophyll *a* concentrations contributed to defining mainly the first axis. These variables were inversely related to the proportion of the *mcyA* genotype strains. The second axis was defined by water temperatures and zooplankton variables versus nitrate concentrations.

In order to determine the relationship between the changes in the proportion of *mcyA* genotype strains and these environmental and biological variables, we performed an SMR analysis. The following equation was obtained: percentage of strains with the *mcyA* genotype = $75.7 - 0.71 \times [\mathrm{SRP}] - 0.024 \times [\mathrm{NO_3}^-] - 0.14 \times \mathrm{Clad} - 1.12 \times 10^{-5} \times$ *P. agardhii* cell density, where [SRP] is the concentration of SRP expressed as micrograms per liter, [$\mathrm{NO_3}^-$] is the concentration of nitrate expressed as milligrams per liter, Clad is the abundance of cladocerans expressed as ind per liter, and the *P. agardhii* cell density is expressed as cells per milliliter. Pearson's R^2 was equal to 63%, with a P value of $<10^{-4}$ for the concentrations of $\mathrm{NO_3}^-$, cladocerans, and *P. agardhii* cells and a P value of <0.05 for the concentration of SRP.

Relationship among changes in the *mcyA* genotype cell density, the MC concentration, and the MC cell quota. The variations in the MC concentration and in the density of *mcyA* genotype cells (defined on the basis of the dynamics of *P. agardhii* cell density and the proportion of the *mcyA* genotype strains) are reported in Fig. 5. The MC concentration was below the detection threshold during the third period, which corresponded to the lowest density of cells with the *mcyA* genotype. During the other periods, a significant positive correlation between the *P. agardhii* cell density and the MC concentration ($R^2 = 0.49$; $n = 45$; $P < 10^{-4}$) was found. This correlation was slightly greater when the *mcyA* genotype cell density and the MC concentration (Pearson's $R^2 = 0.54$; $n = 45$; $P < 10^{-4}$) were considered. From this R^2 value, it appeared that only 54% of the variations in MC concentration could be accounted for by variations in the density of the *mcyA* genotype cells. The highest concentrations of MC (7.4 and 3.5 μg equivalents of MC-LR liter^{-1}, respectively, during periods 1 and 4)

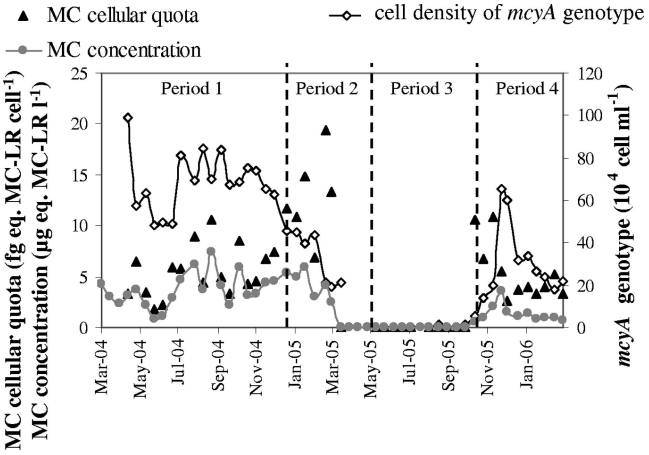

FIG. 5. Changes in the density of *P. agardhii mcyA* genotype cells, the MC concentration, and the MC cell quota from March 2004 to March 2006. eq., equivalents.

were observed (Fig. 5) when the *mcyA* genotype subpopulation reached around 7×10^5 and 3×10^5 cells ml^{-1}.

The variations in the MC cell quota (the ratio between the MC concentration and the density of cells with the *mcyA* genotype) are also reported in Fig. 5. A negative correlation was observed during two periods. Firstly, from winter 2004 to spring 2005 (from the end of period 1 to period 2), the MC cell quota increased simultaneously with a decrease in the density of the *mcyA* genotype cells (Pearson's $R^2 = 0.77$; $n = 10$; $P < 10^{-3}$). Secondly, at the beginning of period 4, the MC cell quota decreased concomitantly with an increase in the *mcyA* genotype cell density (Pearson's $R^2 = 0.65$; $n = 5$; not significant). The ratio was not computed when the MC concentration and/or the *mcyA* genotype cell density was too low (from March to October 2005), as this approach could have produced unrealistically high ratios.

DISCUSSION

Temporal changes in the dynamics of the *P. agardhii* population and in the proportion of cells with the *mcyA* genotype. This 2-year study of the dynamics of the *P. agardhii* population during the perennial bloom in the BNV revealed considerable variation in terms of cell density, as well as the proportion of *mcyA* genotype strains. This perennial *P. agardhii* bloom has been under surveillance since 1999 because of the recreational activities that take place at the BNV (3, 65). So far, the *P. agardhii* population has constituted over 90% of the phytoplankton community, followed by *L. redekei* (accounting for less than 10%) and centric diatoms (about 1%) (3). During this 2-year survey, the dynamics of the *P. agardhii* population in the BNV changed drastically, with a downward trend in *P. agardhii* cell density and a switch of dominant species (e.g., *L. redekei* was dominant in March and April 2005). The decrease in the *P. agardhii* cell density was significantly related to that in the SRP concentration resulting from improved treatment of the incoming watershed surface water in December 2003 (8).

As shown previously, the *Planktothrix* population in the BNV consists of both toxic and nontoxic subpopulations (65), confirming the patchy distribution of *mcy* genes in *P. agardhii* blooms (27). This study revealed that the proportions of strains with the *mcyA* genotype in this *Planktothrix* population were characterized by (i) high values, (ii) a wide range of variation, from 31 to 83%, and (iii) a negative correlation with the *P. agardhii* cell density. The high proportion of potentially toxic clones is consistent with previous data that 88% of *P. agardhii* strains isolated from the same lake and 52% of *P. agardhii* strains isolated from Austrian or Swiss lakes were MC producing (27, 65) and that 100% of the *P. rubescens* filaments isolated contained *mcy* genes (27). When these findings are compared with those available regarding *Microcystis*, the proportion of toxic strains in *Microcystis* populations seems to be lower overall than the proportion of such strains in *Planktothrix* populations. Low percentages of *mcyB* genotype strains (from 1 to 38%) in a natural *M. aeruginosa* population in Lake Wannsee were found previously (26), and the mean proportion of strains of the *mcyB* genotype was stable during the period of seasonal population growth. Roughly the same range of ratios (from 0.5 to 37%) for the *mcyA* subpopulations in two other *M. aeruginosa* populations from Japanese lakes has been estimated previously (66, 67). Finally, a low incidence (<50%) of MC synthetases genes in the *Microcystis* population in Lake Ontario has also been estimated previously (17).

All these data suggest that the proportion of MC producer genotypes in *Planktothrix* blooms is higher than that in *Microcystis* proliferations. Moreover, within the *Planktothrix* genus, the proportions of MC producer genotypes in *P. rubescens* populations are greater than those in *P. agardhii* populations (27). In addition to the trophic statuses of their habitats (eutrophic and hypereutrophic for *P. agardhii* and *M. aeruginosa* versus mesotrophic for *P. rubescens*), these species are also characterized by having different ecological strategies (42). This finding suggests that several environmental parameters may influence the differences observed in the proportions of potentially MC-producing strains of these species.

In our study, both the PCA and the SMR analysis confirmed that there was an inverse relationship between the proportion of the *mcyA* genotype and the *P. agardhii* cell density in the BNV population. They also suggested that the concentrations of some nutrients (phosphorus and nitrogen) may influence the proportion of MC producer genotypes in the *P. agardhii* population. Given that the density of *P. agardhii* cells in the BNV is influenced principally, as in numerous other freshwater ecosystems, by the concentration of SRP (8), it is possible that the impact of environmental factors, such as the concentration of SRP, may act indirectly on the proportion of the *mcyA* genotype via their influence on the *P. agardhii* cell density. Moreover, both the PCA and the SMR analysis revealed a negative correlation between nitrate concentrations and the proportion of *mcyA* genotype strains in this population. However, the SMR analysis revealed that the variations in nitrate concentrations had an impact on the proportion of *mcyA* genotype strains in the *P. agardhii* population only during two short periods (January to March 2005 and February to March 2006) during which high concentrations of NO_3^- and low concentrations of SRP were recorded. The limitation of *Planktothrix* growth by phosphate may explain these findings, which contrast with those of the recent study by Yoshida et al. (67) that revealed a possible impact of nutrient concentrations on

the relative dynamics of MC-producing and non-MC-producing cyanobacterial subpopulations. The data of Yoshida et al. (67) suggest concomitant increases of the proportion of the MC producer *Microcystis* genotype and of the nitrate concentration, which is consistent with previous experimental data from Vézie et al. (60). Therefore, further experimental studies of *P. agardhii* populations are needed to determine the impact of different concentrations of nutrients on the MC-producing and non-MC-producing subpopulations.

By SMR analysis, a significant negative relationship between the proportion of *mcyA* genotype strains and the abundance of cladocerans was also found. However, the variations in cladoceran abundance had only a minor impact on the variations in the proportion of *mcyA* genotype strains, probably due to the small size of the cladoceran population, with the exception of summer 2005, when the *P. agardhii* population was characterized by a low biomass and was not dominant. As cladocerans seem to have less capacity to graze on *Planktothrix* filaments than other phytoplanktonic species (e.g., reference 35), the relationship between cladocerans and the proportion of *mcyA* genotype strains may be indirect, resulting, for example, from the predation of competitive species.

Finally, it appeared that the most important factor having an impact on the proportion of the *mcyA* genotype was variations in *P. agardhii* cell density, which matches previous observations by Janse et al. (18) on members of the *Microcystis* genus. These authors reported a relationship between the proportion of *mcy* genotypes (indirectly assessed by internal transcribed spacer genotyping) and the biomass of cyanobacteria in Dutch lakes. Moreover, Kardinaal et al. (23) showed recently in culture experiments that non-MC-producing *Microcystis* strains are better competitors for light than MC-producing ones. Consequently, the self-shading generated by high biomasses may have an impact on the proportions of MC-producing and non-MC-producing strains in cyanobacterial populations. Experiments to investigate the impact of light and other environmental factors on the cell growth of *P. agardhii* strains need to be performed to find out which MC-producing and non-MC-producing subpopulations have the best fitness.

The relative selection of MC and non-MC producer genotypes seems to depend on multiple factors and processes acting directly or indirectly on these two genotypes and also on other selective traits. A good illustration of this complexity was provided during period 4 of this survey when, in contrast with the 2-year global trend, a concomitant increase in the cell density and in the proportion of *mcyA* genotype strains occurred. This event indicated that under some environmental conditions, the negative relationship between the *mcyA* genotype proportion and the *P. agardhii* cell density can be reversed to become positive. The production of toxins may be an advantage given their potential role in defense against predation or in cell-to-cell communication, for example, at the beginning of a bloom, as has been suggested by Schatz et al. (46, 47).

Relationships among variations in the proportion of the *mcyA* genotype, the MC concentration, and the MC cell quota in the *P. agardhii* population. The regression analysis suggests that only 54% of MC concentration variations can be explained by variations in the density of cells with the *mcyA* genotype. This result means that a high proportion of toxin concentration variations may be attributable to the MC cell quota. This quota ranged from 2 to 19 fg per toxic cell, i.e., it was lower than values found in other studies of *Planktothrix* and *Microcystis* species: 75.6 fg cell^{-1} for *P. agardhii* (1), 100 to 300 fg cell^{-1} (5) and 210 to 1,500 fg cell^{-1} (32) for *P. rubescens*, and 0 to 48 fg cell^{-1} (18) and around 40 fg cell^{-1} (56) for *Microcystis*. Several recent studies have reported that during *Microcystis* spp.-dominated blooms, the MC cell quotas were lower when the biomass was high (18, 22, 61). An inverse relationship between the *mcyA* genotype cell density and the MC cell quota from winter 2004 to spring 2005 and during fall 2005 was also observed in our study. This negative correlation is not surprising given the energy cost of MC production for the cell, as suggested by Kardinaal et al. (23), and the potentially useful roles of MCs, especially as a defense against grazers. The need for a cyanobacterial population to produce MCs can be expected to be greater when cell densities are low. It has also been well established that MC production by the cell depends directly on the growth rate of the cell and indirectly on environmental parameters (5, 15, 29, 30, 37, 50, 65). In the BNV, there was an overall trend over the 2 years of the study for the *P. agardhii* cell density to decrease (8), suggesting that the growth rate of this population was limited.

It is also possible that the relatively weak ($R^2 = 0.54$) relationship between changes in the proportion of *mcy* genotypes and those in MC concentrations may be attributable to the fact that many of the strains identified by molecular methods as being MC producing may not in fact have been producing MC. It has been noticed previously that after some years under laboratory conditions, some strains acquire spontaneous mutations in the *mcy* genes and subsequently become incapable of producing MC (21, 54). Moreover, Kurmayer et al. (27) observed that the percentage of genotypes with inactive *mcy* genes is greater at higher *P. rubescens* cell densities, and Christiansen et al. (10) found that a large proportion of the mutations resulting in the inactivation of MC production are caused by the introduction of an insertion sequence element at different sites. The possibility that this insertion may have happened in our study cannot be ruled out. Any increase in inactive *mcy* genotypes when the *P. agardhii* cell density was high would have led to an underestimate of the cell quota.

Our evaluation of the MC cell quota was performed using the PP2A inhibition assay, but this assay did not provide a quantification of each MC variant separately. Since five variants produced by strains within the *P. agardhii* population from the BNV have been identified previously (3) and have been shown to have different abilities to inhibit PP2A (43), some of the variation in the MC concentration may be explained by fluctuations in the relative levels of production of these different variants. Two recent studies have focused on the factors and processes involved in the production of different MC variants by *Planktothrix* populations. Tonk et al. (55) found experimentally that the production of two MC variants by *P. agardhii* changed with light intensity and that an increase in photon irradiance induced increased production of the more toxic variant. By applying a sequencing approach to the *mcyB* genes of *P. agardhii* and *P. rubescens* strains, it has been demonstrated previously (28) that the biosynthesis of different MC variants depends on the presence of various *mcyB* genotypes.

Finally, the variations in the MC gene expression can probably also account in part for the temporal variations in MC

concentrations. As revealed by Gobler et al. (16), the expression of the MC synthetase gene *mcyE* seems to vary both with the seasons and with environmental processes such as grazing by *Daphnia* sp. However, the level of *mcyE* gene expression is not always consistent with the MC levels measured in the lake.

Methodology. The relative proportion of cells with the *mcyA* genotype within the *Planktothrix* population in the BNV was investigated by using the ΔC_T method applied to the multiplex real-time PCR results. The purpose of this method was to highlight changes in the relative proportion of the *mcyA* genotype within the *Planktothrix* population, rather than to focus on the exact *Planktothrix* cell count. This method, based on the proportions of the PC and *mcyA* genes, was reliable as long as the copy numbers of the targeted genes within the population studied remained constant. Furthermore, this method did not depend on the volume sampled or the quantity of DNA extracted and was not subject to the cell count bias identified in previous real-time PCR studies (26, 58). The highly significant correlation (Pearson's $R^2 = 0.94$; $n = 45$; $P < 10^{-4}$) found between the proportions of the *mcyA* genotype estimated by the GCN method and those estimated by the ΔC_T method demonstrated the validity of the latter method. Independently of the DNA extraction rate, the combination of multiplex real-time PCR with the ΔC_T method was suitable for samples containing small quantities of DNA (with a detection threshold of fewer than 10 cells).

In conclusion, the work reported here revealed an overall negative relationship between the proportion of the *mcyA* genotype and the density of *Planktothrix* cells, except during a short period of intense growth of the *P. agardhii* population. Various environmental parameters were shown to interfere directly or indirectly with changes in the proportions of the *mcyA* genotype. Moreover, during periods of marked changes in the cyanobacterial cell density, a negative relationship between the MC cell quota and the density of *mcyA* genotype cells was found. All these findings suggest that numerous factors and processes may be involved in regulating MC production in cyanobacterial populations, which supports the idea that MCs may possibly be involved in various functions (e.g., cell-to-cell communication, defense against grazing, and nitrogen nutrients). Finally, this study shows the need for experimental studies to elucidate how MC production is controlled in a cyanobacterial population composed of a mixture of MC-producing and non-MC-producing cells and to determine the functional role of MCs.

We also found that the changes in the *mcyA* genotype cell density accounted for only 54% of the variation in the MC concentration from the *Planktothrix* population. From the point of view of possible applications, this finding was particularly interesting in the current context of monitoring programs to predict the toxic risk associated with cyanobacterial proliferation. Our study showed that neither the density of *P. agardhii* cells nor the density of cells with the *mcyA* genotype in this population could have been used to predict the level of toxicity correctly during this 2-year survey of the bloom.

ACKNOWLEDGMENTS

We are grateful to Claude Yéprémian and Charlotte Duval for their help in collecting the cyanobacteria and to Aurélie Ledreux and Arnaud Catherine for PP2A inhibition assays. We thank the UMR-CNRS 7047 (University of Paris 7 and IPGP) and CEREVE (ENPC Paris) laboratories for their help during fieldwork and for providing physical and chemical data. The comments of the two anonymous reviewers were greatly appreciated.

This work was supported by the ECODYN research program (INSU no. 04CV131).

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